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# Optimization of Fermentation Media for Enhanced Amino Acids Production by Bacteria Isolated from Natural Sources

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**Abstract.-** The present study is designed to investigate the enhanced production of amino acids by bacterial strains isolated from different natural sources including sewage water, fresh milk, honey, yoghurt and soil. Sixty five bacterial isolates from these natural sources were isolated and nineteen isolates were found to be producers of amino acids. These isolates were grown in different fermentation media to enhance amino acid production. Out of nineteen, five bacterial isolates were good producers of methionine, cysteine, glutamic acid and valine. On the basis of 16SrRNA nucleotide sequences these organisms were identified as *Bacillus anthracis*, *Bacillus cereus*, *Escherichia coli* H, *Escherichia coli* M2 and *Bacillus* sp. The optimum temperature for the growth of *B. anthracis* and *E. coli* was found to be 37°C, while *B. cereus* and *E. coli* showed optimum growth at 30°C. *Bacillus* sp. showed its optimum growth at 39°C. *B. anthracis*, *E. coli* H and *Bacillus* sp. showed optimum growth at pH 7, while optimum pH for growth of *B. cereus* was 6. *E. coli* M2 showed its maximum growth at 8. The maximum amount of cysteine and methionine produced by *B. anthracis* were 13.28g/l and 12.52 g/l in GM2 and urea based medium UM4 fermentation media, respectively. *B. anthracis* was also capable to produce 9.6g/l of glutamic acid and 4.08 g/l of valine in UM1 medium. *B. cereus* produced maximum cysteine (11.4g/l) and glutamic acid (11.06g/l) in UM1 medium, while maximum amount of methionine (11.2g/l) was produced in UM4 medium. Maximum valine produced was 12.9g/l in GM2 medium. Maximum cysteine and valine produced by *E. coli* were 11.6 g/l and 7.6 g/l in UM1, while maximum amount of methionine and glutamic acid produced was 13 g/l and 10.2 g/l in GM2 medium. The maximum amount of lysine produced was 1.2 g/l in UM2. Maximum amount of cysteine, glutamic acid and valine produced by *E.coli* M2 were 9.7 g/l, 12.5 g/l and 8.24 g/l, respectively in UM1 medium. *E. coli* M2 produced 12.6g/l of methionine in UM2. *Bacillus* sp. S6 produced 10.0 g/l of cysteine in UM2, and 8.12 g/l methionine in GM2. Maximum concentration of glutamic acid and valineproduced was 9.6 and 8.07 g/l in UM1. To conclude the indigenously isolated bacterial isolates can be employed for overproduction of the industrially important amino acids.

**Key words:** Amino acid production, bacteria, glucose based media, urea based media, methionine, glutamic acid, valine, cysteine.

## INTRODUCTION

Amino acids are the most important source of energy for all the living organisms. Besides that these are important for pharmaceutical and chemical industries. Amino acids are crucial for the metabolic activities and play important role in the various physiological processes. Amino acids are the building blocks of proteins, so these amino acids constitute a major part of body. Ninety five percent hormones are amino acids. All neurotransmitters are amino acids in nature.

L-cysteine is an important essential sulfur containing amino acid. It is used in medicines, baking, and as a food additive. In a report published in 1994 five cigarette companies announced that cysteine is one of the 599 additives used in cigarettes and it acts as expectortant and increases glutathione level. Cysteine is used for breaking disulphide bonds in the hair keratin and for permanent wave applications mostly in Asia. It reduces toxic effects of alcohol. Industrially cysteine is synthesized by the hydrolysis of proteins like hair and keratin.

Methionine is one of the three amino acids with body needs to manufacture creatine

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monohydrate, a compound that is essential for energy production and muscle building. Creatine is naturally found in muscle tissue that provides energy needed for contraction and relaxtion of muscles. It also boosts athletic performance during short, intense workouts. Creatine is necessary for all muscular function, and thus supports normal functioning of the heart and circulatory system.

Glutamic acid is a stimulating neurotransmitter in the central nervous system and helps to protect the cardiac muscles in heart patients. Main role of glutamic acid is the stabilization of human androgen receptors and hence regulate antiandrogen activities (Georget *et al*., 2006). Intravenous injection of glutamic acid (monosodium glutamate) enhances the exercise tolerance and heart functions in patients of angina pectoris (Thomassen *et al*., 1991; Zello *et al*., 1995).

Valine is non essential amino acid. On cellular level L-valine is metabolized for fuel that supports muscle recovery after pyhsical exertion. Valine also helps in growth of new tissues. It helps in lean muscle growth along with L-arginine through a synergetic relationship. In patients of sickle cell disease, hydrophilic amino acid glutamic acid is substituted by hydrophobic valine, due to this substitution hemoglobin is not able to fold correctly.

The present study is aimed at isolating and characterizing amino acids producing bacteria from natural sources. Different fermentation media were used with a view to enhance the production of amino acids by these bacterial isolates.

## MATERIALS AND METHODS

*Sample collection*

For isolation of bacteria from different natural sources including milk, honey, yoghurt, soil and sewage water samples were collected in clean, sterilized and capped bottles to avoid any contamination during collection.

*Preparation of sample*

The primary medium used for isolation of bacteria was nutrient agar (2.7 g of agar was dissolved in 100 ml of distilled water). The medium was autoclaved at 121ºC and 15lb pressure for 15min. Then medium was poured in Petri plates in laminar flow to avoid contamination. After solidification plates were ready for use.

*Basal media used for isolation of amino acids producing bacteria*

Table I shows the composition of basal media used for primary screening of bacteria which have the ability to produce amino acids. The bacterial isolates which produced methionine, cysteine, glutamic acid and valine were selected for further investigation.

**Table I.- Composition of basal media used for primary screening of amino acid producing bacterial isolates.**

| Ingredients (g/l) | Basal media | | | |
|---|---|---|---|---|
| | BM1 | BM2 | BM3 | BM4 |
| Glucose | 10g | 1g | 20g | - |
| Peptone | - | 10g | - | 1g |
| Beef extract | 1g | 2g | - | - |
| Yeast extract | - | - | G | 2g |
| NaCl | - | - | - | 2.5g |
| $CaCO_3$ | - | - | 10g | - |

*Morphological and biochemical characterization*

Bacterial isolates were characterized by Gram staining, endospore staining, acid fast staining and motility test to study morphological characteristics. Different biochemical tests including catalase test, oxidase test, gelatin hydrolysis test, starch test, carbohydrate fermentation test, methyl red-Voges Proskauer (MRVP) test, citrate test, urease test, indole test, $H_2S$ test, MacConkey test and ethyl methylene blue (EMB) test were performed to study the physiological characteristics of these amino acid producing bacteria. The isolates were Gram stained. Various biochemical tests were performed according to Cappuccino and Sherman (2001).

*Molecular characterization*

For molecular characterization genomic DNA was extracted as described by Carozzi *et al.* (1991) and the 16S rRNA gene of the bacterial isolates was amplified by PCR using 16S rRNA primers (RS-1; 5′-AAACTC-AAATGAATTGACGG-3′, and RS-3; 5′-ACGGGCGGTGTGTAC-3′) (Rehman *et al*., 2007). PCR was performed by initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 52°C for 40 sec, extension at 72°C for 2 min and a final extension at 72°C for 5 min. The PCR product of

**Table II.- Composition of glucose-based fermentation media used for over-production of amino acids by bacterial isolates.**

| Sr.No | Ingredient(g/l) | GM1 | GM2 | GM3 | GM4 | GM5 | GM6 |
|---|---|---|---|---|---|---|---|
| 1 | Glucose | 0.2g | 0.2g | 20g | 30g | 40g | 30g |
| 2 | $Na_2SO_4$ | 1.0g | - | - | - | | |
| 3 | $KH_2PO_4$ | 1.0g | 0.5g | 1.0g | 3.0g | 0.5g | 2.0g |
| 4 | $MgSO_4.7H_2O$ | 0.1g | - | 0.05g | - | 0.3g | 1.0g |
| 5 | $NH_4Cl$ | 5g | - | 7.0g | 1.0g | | |
| 6 | $NH_4NO_3$ | 1.0g | - | - | - | | |
| 7 | $K_2HPO_4$ | 3.0g | - | - | - | | |
| 8 | Peptone | - | 2.0g | - | - | | |
| 9 | Meat extract | - | 2.0g | - | - | 1.0g | |
| 10 | *$CaCO_3$ | - | 3.0g | - | - | | 20g |
| 11 | $(NH_4)2SO_4$ | - | 2.0g | - | - | 5.0g | 10g |
| 12 | Phenol red | - | 0.015g | - | - | | |
| 13 | $CaCl_2$ | - | - | - | 0.03g | | |
| 14 | NaCl | - | - | - | 1.0g | | |
| 15 | Water | 1000ml | 1000ml | 1000ml | 1000ml | 1000ml | 1000ml |

* GM (glucose based medium). All glucose based media differ from each other slightly in composition, so are named as GM1, GM2, GM3, GM4, GM5 and GM6.

0.5kb was extracted from the gel using Fermentas gel extraction kit (# K0692) and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic Analysis System model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. The 16SrRNA gene sequences were compared with known sequences in the GenBank database to identify the most homologenous sequence.

*Determination of optimum growth conditions*

For optimum growth of the bacterial isolates, two parameters *i.e.*, temperature and pH were considered. For determination of optimum temperature, 5 ml LB broth was added in 4 sets, each of three test tubes, autoclaved and inoculated with 20 µl of freshly prepared culture of bacterial isolate by overnight growth at 37ºC in LB broth. The four sets of tubes were incubated at 20ºC, 30ºC, 37ºC and 45ºC. After an incubation period of 12 h, their absorbance was taken at 600 nm using a lambda 650 UV/V Spectrophotometer (Perkin Elmer, USA). For determination of optimum pH, test tubes having 5 ml LB broth were prepared in 6 sets, each containing 3 test tubes and their pH was adjusted at 5, 6, 7, 8 and 9 and then autoclaved. These tubes were inoculated with 20 µl freshly prepared culture of bacterial isolate. After an incubation period of 12 h, their absorbance was taken at 600 nm.

*Bacterial growth curves*

To determine the growth behavior of the isolates over a period of time, they were grown in LB broth medium. The medium (100 ml) was dispensed in 250 ml flasks and autoclaved at 121$^o$C for 15 min. After the temperature of the medium had lowered to room temperature it was inoculated with 100 µl of log phase growing cells of bacterial isolates. The flasks were incubated at 37$^o$C for 25 h. After every 5 h of incubation, the optical density of culture was measured at 600 nm.

*Fermentation media used for overproduction of amino acids*

The selected bacterial isolates which produced cysteine, methionine, glutamic acid and valine in significant amounts were further proceeded with different fermentation media containing all the essential ingredients required for amino acids biosynthesis (Ali *et al*., 2011). Six fermentation media based on glucose (Table II) were used to enhance the production of amino acids(glucose as a major carbon source) while four media were based on urea as a major nitrogen source (Table III). Two fermentation media were based on industrial wastes like molasses (Table IV). All fermentation media were sterilized at 121ºC and 15 lb pressure for 15 min. After sterilization calcium carbonate was used to keep the pH neutral.

The selected bacterial isolates were

inoculated in 100ml of each fermentation medium in 250 ml Erlenmeyer flasks and incubated at 37±2ºC in shaking incubator at 125 rpm for 96 h. After every 24 h the sample was taken from each flask and production of each amino acid was determined by acid ninhydrin method.

**Table III.- Composition of urea based fermentation media used for enhancement of amino acids production.**

| Sr . No. | Ingredient (g/l) | UM1 | UM2 | UM3 | UM4 |
|---|---|---|---|---|---|
| 1 | Glucose | 5.0g | 5.0g | 3.0g | 5.0g |
| 2 | Urea | 8.0g | 8.0g | 6.0g | 5.0g |
| 3 | $K_2HPO_4$ | - | 0.5g | 0.5g | 0.5g |
| 4 | $KH_2PO_4$ | 5.0g | | 0.5g | |
| 5 | $MgSO_4.7H_2O$ | 0.2g | 0.2g | | 0.2g |
| 6 | $CaCl_2.2H_2O$ | - | - | .05g | |
| 7 | $(NH_4)_2SO_4$ | - | | 3.0g | |
| 8 | EDTA | - | | 0.2g | |
| 9 | Citric acid | - | | 0.1g | |
| 10 | Peptone | 2.0g | | | 0.5g |
| 11 | Meat extract | 2.0g | 2.0g | | |
| 12 | (NH4)3PO4 | - | | | 4.5g |
| 13 | Phenol | 0.015g | | | |
| 14 | Yeast Extract | - | | | 5.0g |

* UM (Urea based Medium)

**Table IV.- Composition of molasses based fermentation media used for over production of amino acids.**

| Sr.No. | Ingredient (g/l) | MM1 | MM2 |
|---|---|---|---|
| 1 | Molasses | 25g | 15g |
| 2 | Meat extract | - | 5.0g |
| 3 | $KH_2PO_4$ | 0.5g | 0.5g |
| 4 | $MgSO_4.7H_2O$ | 0.5g | 0.3g |
| 5 | $K_2HPO_4$ | 0.5g | - |
| 6 | $(NH_4)_2SO_4$ | - | 2.5g |
| 7 | *$CaCO_3$ | 8g | - |
| 8 | NaCl | - | 2.5g |
| 9 | Water | 1000ml | 1000ml |
| | pH | 7.0 | 7.0 |

*MM (Molasses based medium) MM1 and MM2 differ from each other in composition.

*Amino acids analysis*

For the analysis of amino acids 5ml of fermented broth from each medium was taken after every 24 h and centrifuged at 4000rpm (2500xg) for 5 min. Supernatant containing amino acids was separated, which was membrane filtered (0.45µm pore size) to make it cell free. Amino acids were quantitatively determined by acid ninhydrin method (16ml of 0.6M (58.8g/litre) phosphoric acid was mixed with 64ml of glacial acetic acid and 1 g of ninhydrin to prepare 80 ml of ninhydrin reagent; Sigma) in which reaction of amino acids with ninhydrin produces carbon dioxide and aldehyde. Certain amino acids such as ornithine and lysine form colored products when heated with ninhydrin solution (Chinard, 1952).

*Procedure*

Supernatant from each medium (50µl) was taken in capped Pyrex tubes and 550µl of ninhydrin reagent was added to it. The tubes were kept in water bath for one hour at 100°C. Then tubes were cooled at room temperature and 1600µl of glacial acetic acid was added in each tube. Optical density of each sample was taken at respective wavelength for each amino acid, *e.g.*, 600nm for lysine, 365 nm for cysteine, 480 nm for methionine and 510 nm for valine, 340 for tryptophan, 370nm for tyrosine, 470 nm for glutamic acid (Ali *et al*., 2011). Their standard curves of each amino acid with the same procedure were prepared and were used to find out the concentrations of each amino acid produced by bacterial isolates in different fermentation media.

*Statistical analysis*

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken, their mean, and standard error of the mean were calculated.

## RESULTS

In the present study sixty five bacterial isolates were isolated from various sources, among these four isolates were from yogurt, twenty one from soil, ten from milk, five from honey and twenty five isolates from sewage wastewater. Out of sixty five bacterial isolates nineteen were able to produce different amino acids. Of these five bacterial isolates produced cysteine, methionine, glutamic acid and valine in large quantities were selected for further study.

Table V shows the biochemical characteristics of bacterial isolates used in this study.

**Table V.- Morphological and biochemical characteristics of amino acid producing bacterial isolates (A1, A9, H, M2 and S6).**

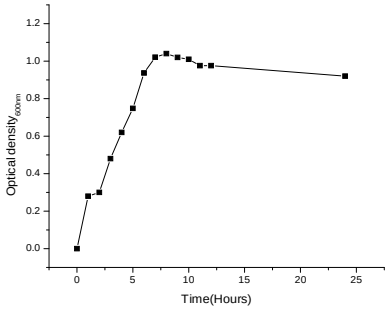
| Tests performed | A1 | A9 | H | M2 | S6 |
|---|---|---|---|---|---|
| Gram's staining | +ve | +ve | -ve | -ve | +ve |
| Endospore staining | +ve | +ve | -ve | -ve | +ve |
| Acid fast staining | +ve | +ve | -ve | +ve | +ve |
| Motility test | +ve | +ve | +ve | +ve | +ve |
| Catalase test | +ve | +ve | +ve | +ve | +ve |
| Urease test | +ve | +ve | -ve | -ve | -ve |
| Gelatin hydrolysis test | +ve | +ve | +ve | +ve | +ve |
| Carbohydrate fermentation test | +ve | +ve | +ve | +ve | -ve |
| Citrate test | +ve | -ve | -ve | -ve | +ve |
| Starch test | +ve | +ve | -ve | -ve | -ve |
| Methyl red-voges proskauer | +ve | +ve | +ve | +ve | -ve |
| Casein hydrolysis test | +ve | +ve | +ve | +ve | -ve |
| Blood agar test | -ve | -ve | -ve | -ve | -ve |
| MacConkey agar test | +ve | -ve | -ve | +ve | +ve |
| Ethyl methylene blue test | -ve | -ve | +ve | +ve | -ve |

+ve, positive; -ve, negative.
A1, *Bacillus anthracis*; A9, *Bacillus cereus;* H, *Escherichia coli* H; M2, *Escherichia coli* M2; S6, *Bacillus* sp.

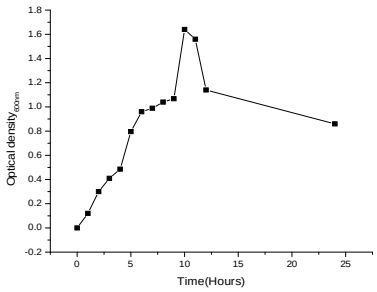
The amplified and sequenced 16S rRNA genes from the local isolates were uploaded to the National Center for Biotechnology Information (NCBI) website to search for similarity to known DNA sequences and to confirm the species of the locally isolated bacteria. The nucleotide sequences after BLAST query revealed that this gene is 99% homologous to *Bacillus anthracis*, 98% homologous to *Bacillus cereus*, 98% homologous to *Escherichia coli* (H), 94% homologous to *Escherichia coli* (M2) and 99% homologous to *Bacillus* sp.. The nucleotide sequences coding for the 16S rRNA gene of bacteria have been submitted to the GenBank database under accession numbers HE 653998, HE 653999, HE 653995, HE 653996, and HE 653997, respectively.

*Growth curves*

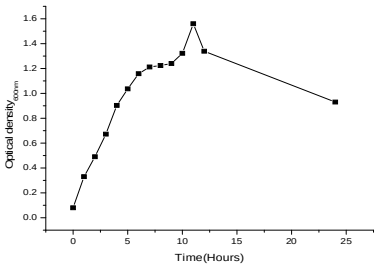
To know the behavior of the amino acids producing bacteria the growth curves were prepared in LB medium and it was found that the lag phase was very short up to 2 h and the log phase was extended upto 12 h in all organisms. After 12 h of incubation growth of the organisms was declined and become stable (Fig. 1).



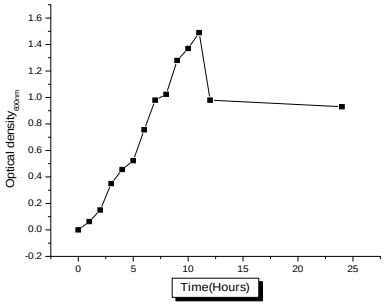

*B. anthracis*A1



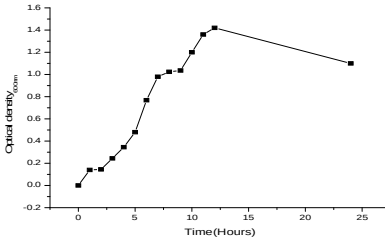

*B. cereus* A9




*E. coli* H




*E. coli* M2




*Bacillus* sp. S6

Fig. 1. Growth curves of the amino acids producing bacteria incubated at their respective temperature for 25 hours.

*Enhancement of amino acid production*

Different fermentation media were used to obtain maximum production of amino acids particularly cysteine, methionine, glutamic acid and valine. Five bacterial isolates which were found to be able to produce these amino acids in significant amount (above 5g/l) were considered as good amino acid producers.

*Qualitative and quantitative analysis of amino acids*

Table VI shows amount of cysteine, methionine, glutamic acid and valine by 5 different isolates. Lysine was produced in small amount by these isolates. Each amino acid production was in one litre of culture medium, which was inoculated by 100µl of inoculums and incubated at 37±2ºC for 24 to 96 h.

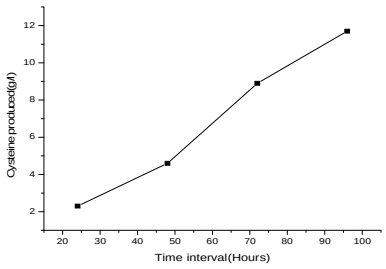
Twelve fermentation media were used to enhance the production of amino acids. Bacterial isolates showed maximum production of amino acids in fermentation media GM2, UMI and UM2.Other fermentation media also proved to be significant in producing amino acids were UM2, UM3 and UM4 (Table VI).

*Amino acids production by bacterial isolates in fermentation media*

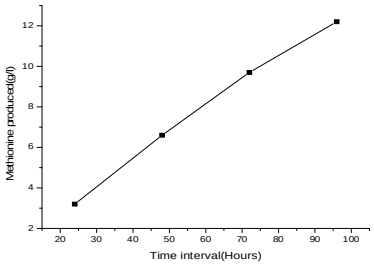
Bacterial isolates showed significant production of amino acids *e.g.*, cysteine, methionine, glutamic acid, valine and lysine in different fermentation media.

Bacillus anthracis (*A1)*

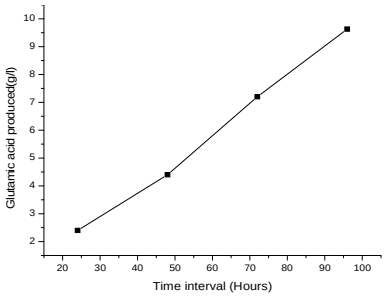
*B. anthracis* A1 was isolated from sewage water and produced methionine, cysteine and glutamic acid in different fermentation media (Fig. 2). The amount of cysteine produced was 13.28g/l in GM2 while no production was seen in GM3 and GM5 (Table VII). Maximum amount of methionine produced by *B. anthracis*A1 was 12.52g/l in fermentation medium of UM4 (Fig. 2) while no production was seen in GM5 and GM6 (Table VII). *B. anthracis* also proved to be a good producer of glutamic acid and maximum amount produced was 9.6g/l in UM1 medium and no concentration was determined in MM2 and GM5 media (Table VII). Valine was produced 4.08g/l in UM1 and least production was 0.3g/l in GM4 medium (Table VII).



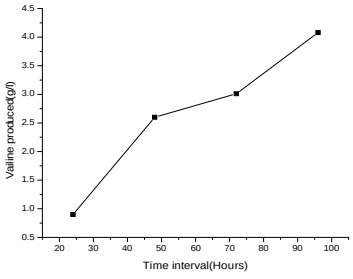

**Cysteine(GM2)**




**Methionine (GM2)**




**Glutamic acid (UM1)**




**Valine(UM1)**

Fig. 2. Time course production of maximum cysteine, methionine, glutamic acid and valine (g/l) in different fermentation media at 37°C by *B. anthracis*A1.

Bacillus cereus (*A9)*

*B. cereus* A9 was isolated from sewage water and produced cysteine, methionine, glutamic acid and valine in different fermentation media. Maximum cysteine was produced 11.4g/l in UM1 medium (Fig. 3) and least production was 0.5g/l in UM4. No production was seen in GM3 and GM5 (Table VII). *B. cereus* A9 produced methionine

**Table VI.- Amount of amino acids (g/l) produced by bacterial isolates in different fermentation media.**

| Sr No | Bacterial isolates | Methionine | Cysteine | Glutamic acid | Valine | Lysine | pH |
|---|---|---|---|---|---|---|---|
| 1 | *B. anthracis* | 12.52(UM4) | 13.28(GM2) | 9.6(UM1) | 4.08(UM1) | 0.7 (UM1) | 7.2,7.2,7.0 |
| 2 | *B. cereus* | 11.2(UM4) | 11.4(UM1) | 11.06(UM1) | 11.1(MM1) | 0.9 (UM1) | 7.2,7.2,7.2 |
| 3 | *E. coli* H | 13(GM2) | 10.4(GM2) | 10.2(GM2) | 7.6(UM1) | 1.2 (UM2) | 7.1,7.2,7.2 |
| 4 | *E. coli* M2 | 12.6(UM2) | 9.7(UM1) | 12.5(GM2) | 8.24(UM1) | 0.5 (UM3) | 6.8,7.2,7.2 |
| 5 | *Bacillus* sp. | 8.12(GM2) | 10.07(UM2) | 9.6(UM2) | 8.07(UM1) | 0.8(UM1) | 7.0,6.9,6.9,7.2 |

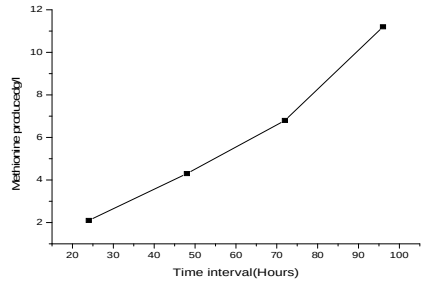
**Table VII.- Production of Cysteine, methionine, glutamic acid, valine and lysine (g/l) by bacterial isolates in different media.**

| Bacterial isolates | Media | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | GM1 | GM2 | GM3 | GM4 | GM5 | GM6 | UM1 | UM2 | UM3 | UM4 | MM1 | MM2 |
| **Cysteine (g/l)** | | | | | | | | | | | | |
| *B. anthracis* | 1.2 | 13.2 | 0 | 6.8 | 0 | 0.9 | 9.5 | 9.8 | 0.9 | 0.8 | 1.1 | 0.7 |
| *B. cereus* | 1.4 | 8.2 | 0 | 10.2 | 0 | 1.1 | 11.4 | 8.6 | 0.7 | 0.5 | 1.4 | 0.6 |
| *E. coli* H | 0 | 6.2 | 0 | 2.2 | 0 | 3.1 | 11.6 | 10.4 | 1.3 | 0.4 | 9 | 0.2 |
| *E. coli* M2 | 0 | 8.2 | 0 | 6.3 | 0 | 2.3 | 9.7 | 9.5 | 0.9 | 0.6 | 0.5 | 0.1 |
| *Bacillus* sp. | 0.4 | 8.7 | 0 | 6.4 | 0 | 0 | 9.5 | 10.0 | 0 | 0.7 | 0.8 | 0 |
| **Methionine (g/l)** | | | | | | | | | | | | |
| *B. anthracis* | 3.3 | 7.9 | 2.1 | 7.9 | 0 | 0 | 7.8 | 7.0 | 1.1 | 12.52 | 3.7 | 0.9 |
| *B. cereus* | 1.2 | 0 | 0.3 | 9.3 | 0 | 0 | 7.7 | 9.5 | 1.7 | 11.2 | 3.72 | 0.7 |
| *E. coli* H | 2.8 | 13 | 2.2 | 11.4 | 1.3 | 0.9 | 12.2 | 11.3 | 0 | 0.3 | 0 | 0 |
| *E. coli* M2 | 0.9 | 3.4 | 2.6 | 11.6 | 1.4 | 0.6 | 11.6 | 12.6 | 0 | 0 | 3.2 | 0.2 |
| *Bacillus* sp. | 0 | 8.12 | 0 | 1.2 | 0 | 1.4 | 7.2 | 7.8 | 0.4 | 0 | 4.0 | 0.5 |
| **Glutamic acid (g/l)** | | | | | | | | | | | | |
| *B. anthracis* | 0.6 | 0.1 | 1.0 | 2.2 | 0 | 1.4 | 9.6 | 7.2 | 0.9 | 8.1 | 3.4 | 0 |
| *B. cereus* | 0.3 | 7.39 | 0.3 | 1.1 | 0 | 1.4 | 11.06 | 4.7 | 0.7 | 0 | 3.3 | 0 |
| *E. coli* H | 0 | 10.2 | 0.1 | 3.4 | 0 | 1.1 | 7.4 | 7.0 | 0.1 | 0 | 1.7 | 0.9 |
| *E. coli* M2 | 0.2 | 3.2 | 0.2 | 1.4 | 0 | 0.3 | 12.5 | 7.1 | 0 | 0 | 3.1 | 0.2 |
| *Bacillus* sp. | 0.7 | 6.8 | 0.1 | 1.0 | 0 | 0.3 | 9.6 | 5.4 | 0 | 0 | 3.5 | 0 |
| **Valine (g/l)** | | | | | | | | | | | | |
| *B. anthracis* | 1.2 | 2.7 | 0.9 | 0.3 | 2.3 | 1.8 | 4.08 | 0.9 | 0.4 | 2.7 | 3.1 | 0 |
| *B. cereus* | 2.1 | 12.9 | 1 | 4.4 | 0 | 2.2 | 5.0 | 4.9 | 0 | 0 | 1.1 | 1 |
| *E. coli* H | 1.12 | 0.2 | 0.9 | 0.3 | 1.15 | 2.2 | 7.6 | 5.3 | 3.1 | 0.5 | 1.9 | 2.1 |
| *E. coli* M2 | 4.1 | 0.9 | 1.8 | 0 | 0 | 0.9 | 8.24 | 6.3 | 0.6 | 1.9 | 2.2 | 1.7 |
| *Bacillus* sp. | 0.6 | 0.3 | 0.5 | 1.1 | 1.3 | 0 | 8.07 | 0.7 | 0.5 | 0 | 0 | 0 |
| **Lysine (g/l)** | | | | | | | | | | | | |
| *B. anthracis* | 0 | 0 | 0 | 0 | 0 | 0 | 0.7 | 0 | 0.1 | 0.1 | 0 | 0 |
| *B. cereus* | 0.2 | 0.1 | 0 | 0 | 0 | 0.4 | 0.9 | 0.2 | 0.1 | 0.2 | 0 | 0 |
| *E. coli* H | 0.1 | 0.6 | 0.1 | 0 | 0 | 0.3 | 0.8 | 1.2 | 0 | 0 | 0.3 | 0.2 |
| *E. coli* M2 | 0.3 | 0.3 | 0.1 | 0 | 0 | 0 | 0.5 | 0.1 | 0.3 | 0 | 0 | 0 |
| *Bacillus* sp. | 0 | 0.1 | 0.1 | 0.2 | 0.3 | 0 | 0.8 | 0.3 | 0 | 0 | 0.4 | 0 |

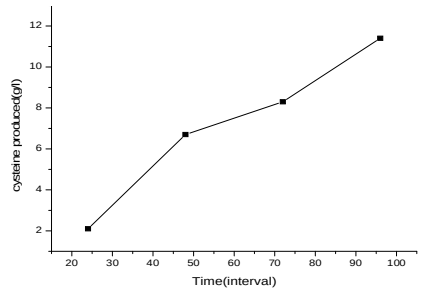
with maximum amount of 11.2g/l in UM4 (Fig. 3) and no production was observed in GM2, GM5 and GM6 (Table VII). Maximum amount of glutamic acid was produced 11.06g/l in UM1 and MM2 showed no concentration was determined in MM2, UM4, and GM5 (Table VIII). Maximum valine produced was 12.9g/l in GM2 and minimum production was 0.4g/l in MM1, MM2 and GM4. No concentration of valine was determined in GM5, UM3 and UM4 (Table VII). Amount of lysine produced was 0.9g/l in UM1 while no significant lysine production was observed in other fermentation media (Table VII).

Escherichia coli *H*

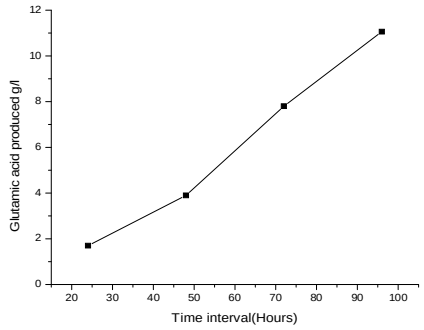
*E. coli* H was isolated from honey sample and showed significant production of methionine, cysteine, glutamic acid and valine in different



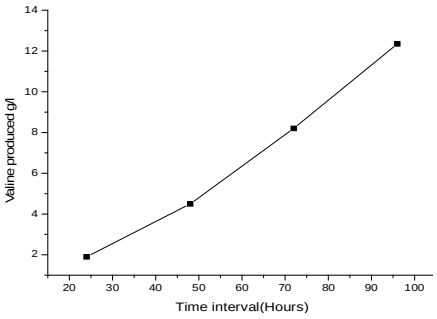

**Methionine (UM4)**




**Cysteine(UM1)**



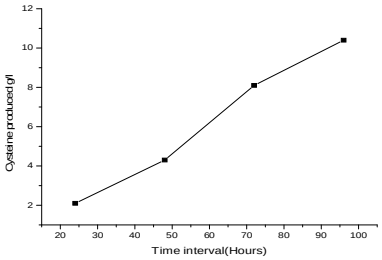

**Glutamic acid(UM1)**



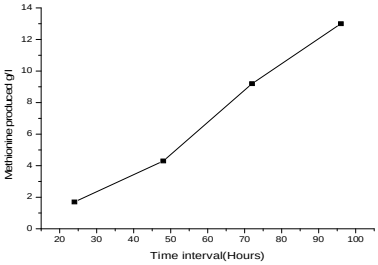

**Valine(GM2)**

Fig. 3. Time course production of cysteine, methionine, glutamic acid and valine (g/l) in different fermentation media at 30°C by *B. cereus* A9.

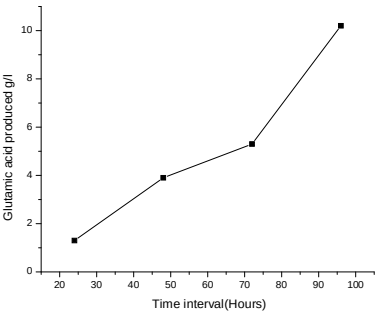
fermentation media. Maximum cysteine produced was 11.6 g/l in UM1 while the minimum amount produced was 0.2g/l in MM2 and no concentration was calculated in GM1, GM3 and GM5 (Table VII). Maximum amount of methionine produced in fermentation medium GM2 was 13 g/l (Fig. 4) and no production was seen in MM1, MM2 and UM3 (Table VII). *E. coli* H produced glutamic acid 10.2 g/l in GM2 (Fig. 4) and no production of glutamic acid was determined in UM4, GM1 and GM5 (Table VII). Maximum valine production was



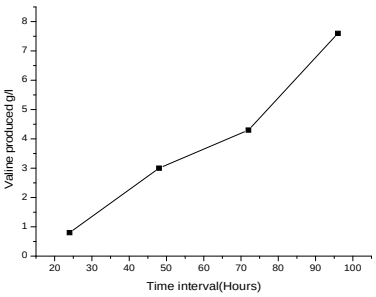

**Cysteine (GM2)**




**Methionine(GM2)**




**Glutamic acid(GM2)**




**Valine(UM1)**

Fig. 4. Time course production of maximum cysteine, methionine, glutamic acid and valine (g/l) in different fermentation media at 37°C by *E. coli* H.

calculated in UM1 (7.6 g/l) and minimum production was determined as 0.2 g/l in GM2 (Table VII). The maximum amount of lysine produced was 1.2 g/l in UM2 (Table VII).

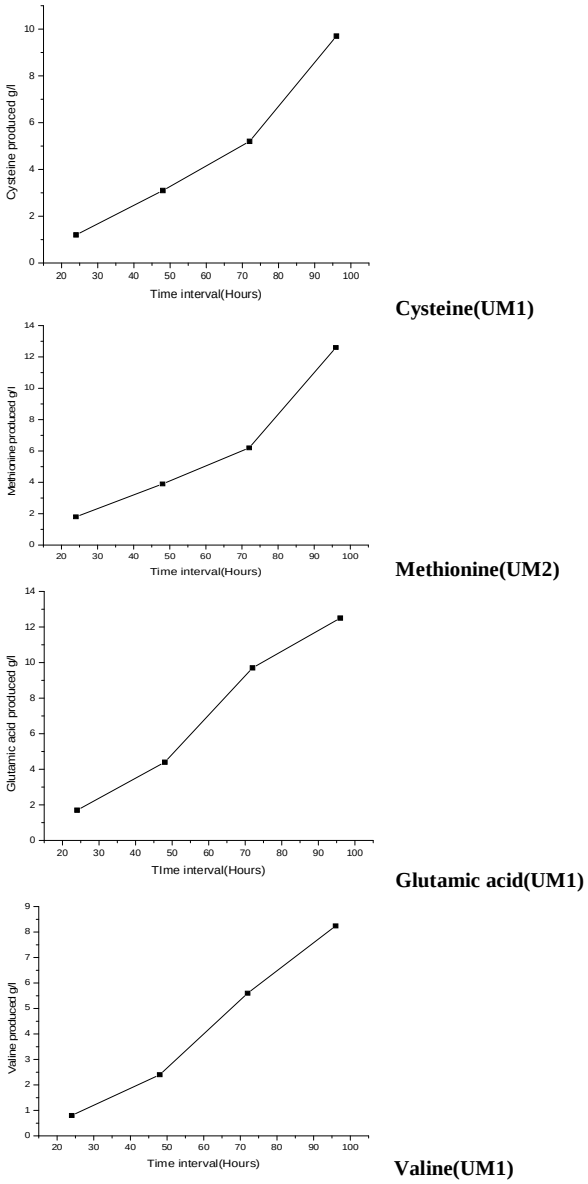



Fig. 5. Time course production of maximum cysteine, methionine, glutamic acid and valine (g/l) in different fermentation media at 30°C by *E. coli* M2.

Escherichia coli *M2*

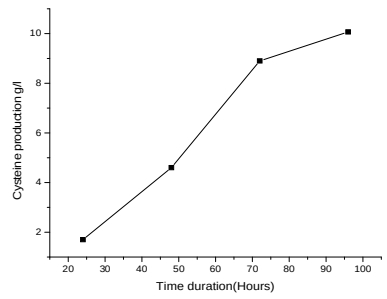
*E. coli* M2 was isolated from fresh milk and showed good production of methionine, cysteine, glutamic acid and valine in different fermentation media except lysine which was produced insignificantly by this bacterium. Maximum amount of cysteine produced was 9.7 g/l in UM1 and minimum production was 0.1 g/l in MM2. No concentration was estimated in GM1, GM3 and GM5 (Table VII). *E. coli* M2 produced maximum amount of methionine 12.6g/l in UM2 (Fig. 5) and minimum of 0.2g/l in MM2 while on concentration was determined in UM3 and UM4 (Table VII). Maximum amount of glutamic acid produced in UM1 was 12.5 g/l (Fig. 5) and no production was determined in UM3, UM4 and GM5 (Table VII). Maximum valine production was 8.24g/l in UM1 while GM4 and GM5 showed no production (Table X). *E. coli* produced lysine 0.5g/l in UM3 while no concentration was determined in GM4, GM5, GM6, UM4, MM1 and MM2 (Table VII).

Bacillus *sp. (S6)*

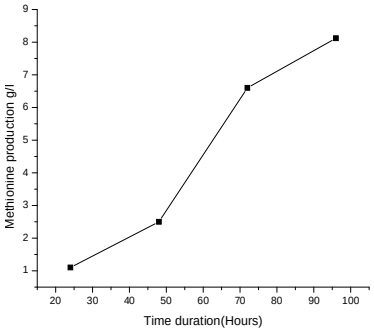
*Bacillus* sp. S6 was isolated from soil sample and produced cysteine, methionine glutamic acid and valine in different fermentation media. *Bacillus* sp.S6 produced 10.0 g/l of cysteine in UM2 medium (Fig. 6) and minimum amount of amino acid produced was 0.4 g/l) in GM1 (Table VII). Maximum production of methionine determined was 8.12 g/l in GM2 (Fig. 6) and no production was observed in GM1, GM3, GM5 and UM4 (Table VII). Maximum concentration of glutamic acid produced was 9.6g/l in UM1, while UM3, UM4, MM2 and GM5 showed no production (Table VII). Maximum production of valine was 8.07g/l in UM1 and no concentration was determined in UM4, MM1, MM2 and GM6 (Table VII). Only 0.8 g/l lysine was produced in UM1 medium while no other medium supported the significant production of lysine (Table VII).

## DISCUSSION

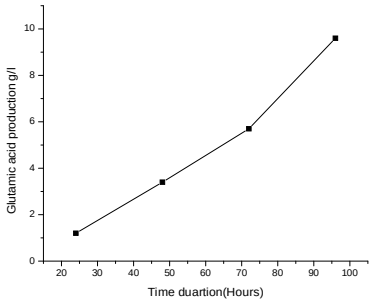
In the present study main emphasis was to isolate bacterial strains from natural sources and enhance the production of amino acids in different fermentation. These were isolated from sewage water, soil, honey, yoghurt and fresh unpasteurized milk. Different fermentation media were used to isolate good producers of amino acids. Parameters followed during isolation of pure cultures were



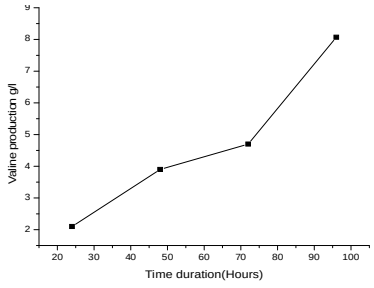

**Cysteine(UM2)**




**Methionine(GM2)**




**Glutamic acid(UM1)**




**Valine(UM1)**

Fig. 6. Time course production of cysteine, methionine, glutamic acid and valine (g/l) in different fermentation media at 39°C by *Bacillus* sp.S6.

temperature, pH, different fermentation media, water quantity and aeration. Incubation time for the bacterial isolates is also very crucial for their growth and amino acid production. These parameters have great impact on the production of amino acids. The optimum temperature for all the strains ranged from 30°C to 39°C and optimum pH for growth ranges 5 to 9. Five bacterial isolates designated as A1, A9, H, M2 and S6 were found to be good producers of amino acids *i.e.*, they produced amino acids above 5g/l. Enterobacteriaceae, Bacillaceae and Pseudomonadaceae are three bacterial families which are considered as good producers of amino acids (Aida, 1986).

Twelve different fermentation media were used for the over production of amino acids by these strains. It is natural ability of most of the bacteria to synthesize amino acids but due to presence of feed back mechanism in these bacteria, the production of amino acid is restricted to a certain low extent. This feedback inhibition in bacteria keeps check on accumulation of intermediate metabolites in the cell (Nakamori *et al.*, 1999). In feed back mechanism only when the end products are in large quantity then the first enzyme of biosynthetic pathway is inhibited.

Synthesis of amino acids is very important for cell survival. Bacteria use significant amount of energy to synthesize the enzymes needed for the pathway that leads towards amino acid biosynthesis through different reactions. Bacteria usually produce amino acids at different rate. Some bacteria are very good producers of amino acids. There are three procedures through which the amino acid producing ability of bacteria can be enhanced, through mutagenesis, genetic engineering and by the use of different fermentation media whose constituents play an important role for growth of bacteria and the production of different amino acids. These fermentation media differ from each other on the basis of their basic constituents which are used as important source for the production of a specific amino acid (Das *et al.*, 1995; Nadeem and Ahmed, 1999).

As the population of world is increasing at an alarming rate, there is ever increasing demand of food for the consumption that has increased the use of amino acids also, has resulted in the demand of amino acids during later half of $20^{th}$ century. In the present study twelve fermentation media were used to enhance the growth and ability of amino acid production of bacterial isolates. These fermentation media were glucose, urea, and molasses based with carbon and nitrogen as key elements.

For the industrial production of most L-amino acids the fermentation method is used. Coryneform bacteria have played very important role in fermentation industry for the production of amino acids. Detailed information on the metabolic pathways, their regulation and mutations can lead to the increased production of amino acids (Wang *et al*., 2002). Different Coryneform sp. synthesize different amino acids through metabolism. For example *Corynebacterium glutamicum* produces serine, lysine and glutamate which are used in pharmaceutical industries (Jo *et al.*, 2006).

Both enzymatic and fermentative processes are used for the production of cysteine in industries. Through microbial fermentation L-cysteine has been developed which is more economical and is safe for environment. Now a days *Corynebacterium glutamicum* and *E. coli* are used for the production of many amino acids in amino acid industry, both have same biosynthetic pathway for L-cysteine. In both bacteria L-cysteine degrading enzymes and L-cysteine exporting proteins are studied (Quig, 1998).

Cysteine production by bacterial isolates in different fermentation media has also been reported by Ali *et al.* (2011). In the present work bacterial strain (H) which was isolated from honey and identified as *E.coli* produced 10.4g/l of cysteine in fermentation media GM2. GM2 is glucose based medium. The cost of this medium is quite economical as compared to the cost of 10g of cysteine commercially produced (Ali *et al*., 2011). Another isolate from milk (M2) which was isolated from milk and identified as *E. coli* also produced 9.7g/l in fermentation media UM1, which is urea based medium. *Bacillus* sp. from soil and *B. cereus* isolates from sewage water also proved to be an important cysteine producing source. By using fermentation media with different natural constituents the production of cysteine can be enhanced through these strains in future. The producer strains for this amino acid are isolated from natural sources (sewage water, soil, milk, honey and yoghurt).

One of the essential amino acids that body can not synthesize is methionine so its intake in the form of protein diet is necessary for the proper body functions. L-methionine has wide applications in industry, pharmaceuticals and as food additives. It is also used as a methylatingagent. Mainly methionine is produced by the breakdown of racemic DL-methionine other method used for its production is the extraction method, in which by the hydrolysis of protein, methionine is produced and extracted. Different strains of *E. coli* and *Bacillus* sp. have been useful for the production of this amino acid through fermentation media (Karlstrom, 1965). Shimizu *et al.*(2008) studied that methionine is required in the body for the production of two important amino acids cysteine and taurine (both enhance the natural ability of the body to eliminate toxins, make the body tissues healthy and support cardiovascular tissues).

Bacterial strains from sewage water (A1) and (A19) showed significant production of methionine. Bacterial isolate (H) isolated from honey gave maximum production of methionine 13g/l .This strain was identified as *E. coli* on the basis of ribotyping. The amount produced was in fermentation medium GM2 which has glucose as major constituent. The price of 10g of methionine including 16% GST is Rs. 2134/- which is more expensive (Ali *et al*., 2011), while the media contains the constituents which are not expensive. Commercial production of methionine can be increased by the use of this medium.

Glutamic acid an important component of food additive "monosodium glutamate" is now a days produced by bacterial or microbial fermentation. The bacteria are grown in nutrient medium in which glutamic acid is excreted into the medium through the bacterial cell membrane. Then the glutamic acid is separated from the fermentation broth by different techniques as by filteration, concentration, acidification, and crystallization (Grattini, 2000). Glutamic acid bacteria mostly belong to the genus *Corynebacterium*, but *C. glutamicum* proved to be the most effective for production of glutamic acid (Kinoshita, 1999).

Nature of carbon and energy source plays important role for fermentative process, sugars are the main source but other organic compounds are also used Stanier *et al.* (1987). Extracellular secretion of glutamic acid by *B. cereus* has been reported by Ghosh and Sen (1996). In this recent work bacterial strain (M2) isolated from milk which was identified as *E. coli* gave the maximum

production of glutamic acid 12.5g/l in UM1 fermentation medium which is urea based medium and provided a source of urea that is an important organic compound for the production of this amino acid. This medium is also not very costly and is effective to use for commercial production of glutamic acid.

Being an essential amino acid valine is taken through foods containing it. Valine synthesis in plants takes place via several steps which start from pyruvic acid. The initial steps of the pathway also lead to the formation of leucine. Along with glutamate, alpha ketovalerate which is an intermediate undergoes reductive amination. In valine biosynthesis enzymes involved are, aceto lactate synthase, aceto hydroxy acid isomeroreductase, dihydroxy acid dehydratase and valine aminotransferase.

In recent work bacterial isolate (S6) from soil was identified as *Bacillus* sp. proved to be a significant producer of valine (8.07g/l) in UM1 medium. A *Bacillus* sp. has been identified as producer of valine (Chattopadhyay and Banerjee, 1978). Along with this another isolate from milk(M3) identified as *Bacillus* sp. produced valine as 9.36g/l in GM2 that is glucose based medium. As these species are found everywhere in water, air, soil so these can be a better source for production of this essential amino acid.

It is evident that the microbial fermentation process can be used commercially for the production of amino acids at very economical rates by using locally isolated strains from natural sources. The fermentation media used for over production of these amino acids have shown great results as the isolated bacterial strains produced the amino acids particularly cysteine, methionine, glutamic acid and valine in fair amount. The natural sources including sewage water, soil, milk, honey and yoghurt which are rich in microflora can provide bacterial strains for the production of amino acids by fermentation on commercial scale to meet the local demand in the country.

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